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Alterations in the splicing patterns of key regulatory genes are likely to play an important role in oncogenesis. The ASF/SF2 protein is one of a family of SR splicing factors that have been shown to regulate splice site choice *in vitro*. We have mapped the ASF/SF2 gene to 17q21.3-q22 in humans, and close to the *Ovum mutant* locus on chromosome 11 in mice. Our current objective is to examine the role of the ASF/SF2 gene in development and oncogenesis by observing the effects of disrupting the gene in mice. We have generated seven embryonic stem (ES) cell lines that are heterozygous for a deletion of the ASF/SF2 gene. Chimeric mice have been generated for four of these cell lines, but we have not yet achieved germ line transmission of the ASF/SF2 mutation. It is currently unclear whether this result is due to difficulties in the handling and/or injection of the ES cells, or is due to haploinsufficiency of the ASF/SF2 gene.

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5. Introduction.

Alternative mRNA splicing plays an important role in development and differentiation; many transcripts are spliced differently in distinct cell types and tissues (for reviews see Smith et al., 1989; McKeown, 1992; Hodges and Bernstein, 1994). Many alternatively spliced mRNAs encode proteins that act in transcriptional regulation, in signal transduction, and in the contacts of cells with each other and with the extracellular matrix. Neoplastic growth can result from alteration of each of these processes, and for several genes that function in the development and differentiation of cells and tissues, novel splice variants have been identified from tumors. Transcripts that are differently spliced in tumors include mRNAs encoding fibronectin (Oyama et al., 1989; Oyama et al., 1990), estrogen receptor (reviewed in McGuire et al., 1991), and the cell suface glycoprotein CD44 (Arch et al., 1991; Gunthert et al., 1991; Matsumura et al., 1992). The observation that differently spliced mRNAs exist for these genes in cancer suggests that alteration(s) in the splicing machinery is involved in one or more stages in oncogenesis. An understanding of the relationship between alternative splicing and oncogenesis may have profound implications for the prevention, diagnosis and treatment of many cancers, including breast cancer, and may provide insights into normal development as well.

While examples of alternatively spliced transcripts abound, relatively little is known about the mechanisms involved in regulating the use of alternative splice sites. Both constitutive and alternative splicing occurs on spliceosomes, which are complex particles composed of small nuclear ribonucleoproteins, or snRNPs, and non-snRNP proteins (reviewed in Morre et al., 1993; Newman, 1994). The SR family of non-snRNP splicing factors are characterized by the presence of an RNA recognition motif and a serine and arginine rich (SR) domain (reviewed in Norton, 1994; Horowitz and Krainer, 1994). SR proteins are required at early stages of spliceosome assembly, have distinct but overlapping specificities for different pre-mRNAs, and can alter splice site choice (reviewed in Norton, 1994; Horowitz and Krainer, 1994). These observations suggest that SR proteins are involved in the regulation of alternative splicing *in vivo*. One of the best characterized SR proteins, ASF/SF2, is the focus of this report.

I am taking two experimental approaches to understand better the function of ASF/SF2 *in vivo*. First, I have mapped the gene in mice and humans, to assess potential associations of the gene with classical mouse mutations or human genetic diseases. Second, I am attempting to generate a mice that carry mutant alleles of the ASF/SF2 gene, to determine the significance of its expression to normal and abnormal development.

6. Body.

A. Chromosomal mapping of SR protein genes.

The first specific aim of this research project was to complete the mapping of the ASF/SF2 gene. This objective has been accomplished, and a paper describing the results is in press (Bermingham, et al., 1995). Initial mapping of the ASF/SF2 gene in humans raised the possiblity that it could be a candidate gene for the BRCA1 locus. While the ASF/SF2 gene lies near the BRCA1 locus, cloning of the BRCA1 gene (Miki et al., 1994), demonstrates that ASF/SF2 and BRCA1 are distinct genes. Interstitial deletions of chromosome 17 have been observed in some breast tumors (Cropp et. al., 1993). The loss of a copy of the ASF/SF2 gene could play a role in tumorigenesis if it led to the aberrant splicing of pre-mRNAs from BRCA1 or other gene(s) that function in growth control.

In mice, we have mapped the ASF/SF2 gene to mouse chromosome 11, near the marker D11Mit38 (Bermingham et al., 1995). These results indicate that the ASF/SF2 gene is one of the closest markers to the *Ovum mutant* (*Om*) locus yet identified. *Om* is responsible for a genetic behavior of the DDK inbred mouse strain: DDK females are fertile when mated to DDK males, but are sterile or semi-sterile when mated to males of other inbred strains; DDK males are fertile when mated to females of other inbred strains (Wakasugi, 1974). Analysis of backcross mice (Sapienza et al., 1992) and recombinant inbred mice (Baldacci et al., 1992) placed the *Om* locus on mouse chromosome 11. ASF/SF2 was mapped using backcross mice between the C57BL/6 and DDK strains that were previously described in Sapienza et al., (1992) to locate ASF/SF2 more precisely, and to determine if it could be a candidate gene for the *Om* locus. These results are summarized in figure 1. While the ASF/SF2 gene probably lies distal to *Om*, the precise location of *Om* cannot be determined in crosses, because it is incompletely penetrant. Therefore, our results do not exclude ASF/SF2 as a candidate gene for *Om*.

The proximity of the Sfrs1 and *Om* loci is interesting for two reasons. First, while the ASF/SF2 gene probably lies distal to *Om*, these results do not exclude it as a candidate gene for *Om*. Recent pronuclear and cytoplasmic transplantation experiments (Babinet et al., 1990; Renard et al., 1994) indicate that the lethality of embryos from DDK females bred to males from other strains is due to an incompatability between the cytoplasm of DDK eggs and the paternal genome, and that this incompatability is mediated by RNA(s). Perhaps these RNA(s) could interact with ASF/SF2 protein. Second, the effect of the *Om* locus has been hypothesized to result from a reversal of imprinting at the *Om* locus in the DDK strain (Sapienza et al., 1992). Genomic

imprinting is a little-understood phenomenon that may play important roles in normal development, as well as the etiology of some tumors (for reviews, see Tycko, 1994; Efstratiadis, 1994). Recently two splicing factors, snRNP associated protein N, and U2afbp-rs, have been shown to be imprinted (Leff et al., 1992; Hayashizaki et al., 1994a). If the effects of *Om* reflect the imprinting of a region of DNA on chromosome 11, then perhaps the ASF/SF2 gene is imprinted as well. Mice that are paternally or maternally disomic for chromosome 11 are viable and fertile (reviewed in Cattenach and Beechey, 1990), suggesting that any potential imprinting of ASF/SF2 and/or *Om* is without *gross* phenotypic consequence. However, if imprinting of the ASF/SF2-*Om* region differs between strains or individuals, or alternatively, if the effects of this imprinting are incompletely penetrant, then it could have escaped detection. By acting on genes that encode splicing factors, the imprinting machinery could post-transcriptionally control the expression of non-imprinted genes.

B. Targeted mutagenesis of the ASF/SF2 gene.

The basic procedures for generating mutations in mice by homologous recombination are extensively documented (Joyner, 1993; Wassarman and DePamphilis, 1993; Hogan et al., 1994). Briefly, the procedure consists of the following steps: 1) A targeting construct is built that will undergo homologous recombination with the gene to be disrupted. 2) Mutations are produced in embryonic stem (ES) cells in culture by homologous recombination of the target gene with the introduced DNA. 3) Mutant ES cells are introduced into host blastocysts to that are in turn introduced into pseudopregnant mice and allowed to develop into chimeric mice. 4) The chimeric mice are bred to determine if the ES cells contribute to the germ line of the chimera. If so, mutations that they carry can be transmitted to future generations.

Table I summarizes the progress made so far in producing mice that carry a disrupted ASF/SF2 allele. I have generated seven new ES cell lines that are heterozygous for a deletion of the ASF/SF2 gene. Five of these cell lines have been injected into blastocysts and implanted. Chimeric mice have been obtained from four of the five cell lines. Most of these mice have been bred, but so far, none has produced ES cell-derived progeny. There are two possible reasons for this failure. First, we may have encountered technical problem(s) that will be solved with additional injections, perhaps with new heterozygous ES cell lines. Second, the ASF/SF2 gene may be haploinsufficient in one or more cell types. Currently, we cannot distinguish between these possiblities. I plan three approaches to address this problem. First, I will perform additional injections with the current cell lines and with control cell lines to resolve any technical difficulties with the handling or injection of the ES cells. Second, I will use a polymorphism in the mouse

ASF/SF2 gene (Bermingham, et al., 1995) to determine if maternal and paternal copies of the gene are differentially methylated. Such differential methylation is an indication that the gene is imprinted. If so, one explanation for the difficulties in generating is that the targeting vector disrupted the active copy of the gene. Third, I will generate additional ES cell lines, either using a modification of the current targeting vector, or one that will retain a funtional ASF/SF2 gene that can be removed by tissue or stage-specific expression of the Cre recombinase (for review of this technology, see Chambers, 1994).

7. Conclusions

1.   The ASF/SF2 gene is distinct from the BRCA1 gene, although this observation does not exclude the possiblity that ASF/SF2 plays a role in the etiology of breast cancer by regulating the expression of BRCA1 or other growth control gene(s).

2.   The ASF/SF2 gene is likely to be located distal to the *Ovum mutant* (*Om*) locus in mice, but uncertainties in the position of Om due to incomplete penetrance do not exclude ASF/SF2 as a candidate gene for *Om*.

3.   Seven new embryonic stem cell lines have been isolated that are heterozygous for deletions of the ASF/SF2 gene. Five of these cell lines have been injected into blastocysts, and chimeric mice have been obtained from four lines. However, no germline transmission of the offspring have been observed. At the present time we cannot distinguish between a technical problem with the handling/injections of the ES cells and haploinsufficiency and/or imprinting of the ASF/SF2 gene.

8. References.

Arch, R., Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P. and Zoller, M. (1991). Participation in normal immune responses of a metastasis-inducing splice variant of CD44. Science 257: 682-685.

Babinet, C., Richoux, V., Guénet, J-L., and Renard, J-P. (1990). The DDK inbred strain as a model for the study of interactions between parental genomes and egg cytoplasm in mouse preimplantation development. Development Suppl. 81-87.

Baldacci, P.A., Richoux, V., Renard, J-P., Guénet, J-L., and Babinet, C. (1992). The locus *Om*, responsible for the DDK syndrome, maps close to *Sigje* on mouse chromosome 11. Mamm. Genome 2: 100-105.

Bermingham, J.R., Jr., Arden, K., Naumova., A.K., Sapienza, C., Viars., C.S., Fu, X-D., Khotz, J., Manley, J.L., and Rosenfeld., M.G. (1995). Chromosomal localization of mouse and human gene encoding the splicing facors ASF/SF2 (SFRS1) and SC-35 (SFRS2). Genomics, in press.

Cattanach, B.M., and Beechey, C.V. (1990). Autosomal and X-chromosome imprinting. Development Suppl. 63-72.

Chambers, C.A. (1994). TKO'ed: Lox stock and barrel. BioEssays 16: 865-868.

Cropp, C.S., Campeme, M-H., Liderau, R., and Callahan, R. (1993). Identification of three regions on chromosome 17q in primary human breast carcinomas which are frequently deleted. Cancer Res. 53: 5617-5619.

Efstratiadis, A. (1994). Parental imprinting of autosomal mammalian genes. Curr. Opin. Genet. Dev. 4: 265-280.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haußmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. (1991). A new variant of glycoproteinCD44 confers metastatic potential to rat carcinoma cells. Cell 65: 13-24.

Hayashizaki, Y., Shibata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuizumi, H., Muramatsu, M., Komatsubara, H., Shiroshi, T., Moriwaki, K., Katsuki, M., Hatano, M., Sasaki, H., Ueda, T., Mise, N., Takagi, N., Plass, C., and Chapman, V. (1994a). Identification of

an imprinted U2af binding protein related sequence on mouse chromosome 11 using the RLGS method. Nature Genetics 6: 33-40.

Hayashizaki, Y., Hirotsune, S., Okazaki, Y., Shibata, H., Akasako, A., Muramatsu, M., Kawai, J., Hirasawa, T., Watanabe, S., Shiroshi, T., Moriwaki, K., Taylor, B.A., Matsuda, Y., Elliott, R.W., Manly, K.F., and Chapman, V. (1994b). A genetic linkage map of the mouse using restriction landmark genomic scanning (RLGS). Genetics 138: 1207-1238.

Hodges, D., and Bernstein, S.L. (1994). Genetic and biochemical analysis of alternative RNA splicing. Adv. Genet. 31: 207-281.

Hogan, B., Beddington, R., Constantini., F., and Lacy, E. (1994). Manipulating the Mouse Embryo. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Horowitz, D.S., and Krainer, A.R. (1994). Mechanisms for selecting 5' splice sites in mammalian pre-mRNA splicing. Trends Genet. 10: 100-106.

Joyner, A.L., ed. (1993). Gene Tartgeting: A Practical Approach. Oxford University Press, Oxford, UK

Leff, S.E., Brannan, C.I., Reed, M.L., Ozçelik, T., Franke, U., Copeland, N.G., Jenkins, N.A. (1992). A candidate mouse model for Prader-Willi syndrome which shows an absence of Snrpn expression. Nature Genetics 2: 259-263.

Matsumura, Y., and Tarin, D. (1992). Significance of CD44 gene products for cancer diagnosis and disease evaluation. Lancet 340: 1053-1058.

McGuire, W.L., Chamness, G.C., and Fuqua, S.A.W. (1991). Estrogen receptor variants in clinical breast cancer. Mol. Endo. 5: 1571-1577.

McKeown, M. (1992). Alternative mRNA splicing. Ann. Rev. Cell Biol. 8: 133-155.

Miki, Y., and 44 other authors. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266: 66-71.

Moore, M.J., Query, C.C., and Sharp, P.A. (1993). Splicing of precursors to mRNA by the spliceosome. in The RNA World Pp. 303-357. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Newman, A.J. (1994). Pre-mRNA splicing. Curr. Opin. Genet. Dev. 4: 298-304.

Norton, P.A. (1994). Alternative pre-mRNA splicing: factors involved in splice site selection. J.Cell Sci. 107: 1-7.

Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. (1989). Deregulation of alternative splicing of fibronectin pre-mRNA in malignant human liver tumors. J. Biol. Chem. 264: 10331-10334.

Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. (1990). Oncodevelopmental regulation of the alternative splicing of fibronectin pre-messenger RNA in human liver lung tissues. Cancer Res. 50: 1075-1078.

Renard, J-P., Baldacci, P., Richoux-Duranthon, V., Pournin, S., and Babinet, C. (1994). A maternal factor affecting mouse blastocyst formation. Development 120: 797-802.

Sapienza, C., Paquette, J., Pannunzio, P., Albrechtson, S., and Morgan, K. (1992). The polar-lethal *Ovum Mutant* gene maps to the distal portion of mouse chromosome 11. Genetics 132: 241-246.

Smith, C.W.J., Patton, J.G., and Nadal-Ginard, B. (1989). Alternative splicing and the control of gene expression. Ann. Rev. Genet. 23: 527-577.

Tycko, B. (1994). Genomic imprinting: mechanism and role in human pathology. Am J. Pathol. 144:431-443.

Wakasugi, N. (1974). A genetically determined incompatability system between spermatozoa and eggs leading to embryonic death in mice. J. Reprod. Fert. 41: 85-96.

Wassarman, P.W., and DePamphilis, M.L., eds. (1993). Guide to Techniques in Mouse Development. Academic Press.

9. Appendix

CENTROMERE

```
        Mpmv2      D11Mit5
                            |

        5.8 cM              |

                            |
                            |_ Om

        D11Mit66            |

        4.8 cM              |

                            |

  ASF/SF2    D11Mit38       |

        21.1 cM

        D11Nds2
```
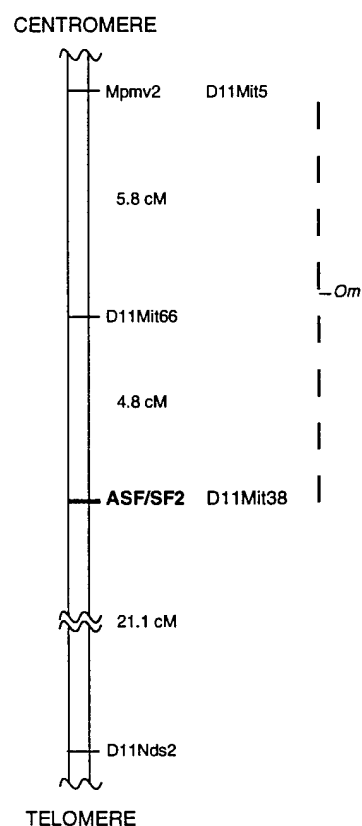
TELOMERE

FIGURE 1: Linkage of Sfrs1 (ASF/SF2) to nearby mouse chromosome 11 markers. (DDK♀ X C57BL/6♂)F1♀ X DDK♂ and (C57BL/6♀ X DDK♂)F1♀ X DDK♂backcross mice were scored for recombination between genetic markers near *Om*, and the resulting female-specific recombination distances between the markers are shown in the figure in centimorgans (cM). These results place the ASF/SF2 gene very close to the marker D11Mit38. The location of *Om* on the map is depicted as a dashed line, because it is incompletely penetrant.

Table 1. Summary of Current ASF/SF2 Knockout Results.

	EXPERIMENT 1	EXPERIMENT 2	TOTAL
ES Cells			
# cell lines screened:	100	162	262
# ASF/SF2 +/- cell lines:	1	7	6 (2.3%)
# cell lines injected:	1	5	6
Mice			
#chimeric mice:	7	16	23
% chimerism:	20-60	20-80	
Sex ratio bias?	yes	yes	
Germline transmission?	no	not yet	